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Steroid Requirement for Androgen Receptor Dimerization and DNA Binding

MODULATION BY INTRAMOLECULAR INTERACTIONS BETWEEN THE NH_2-TERMINAL AND STEROID-BINDING DOMAINS*

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Infection of *Spodoptera frugiperda* Sf9 insect cells with recombinant human androgen receptor (AR) baculovirus results in expression of a 118-kDa phosphoprotein that displays high affinity androgen binding and androgen-dependent targeting to the nucleus. Using the DNA mobility shift assay, specific *in vitro* binding of full-length AR to androgen response element DNA (ARE) requires intracellular hormone exposure. The ability of a variety of steroids to induce ARE binding paralleled their transcriptional potential. Certain antihormones, cyproterone acetate and RU486, promote ARE binding, but a pure antiandrogen, hydroxyflutamide, inhibits AR binding to ARE DNA. AR dimerization requires incubation of recombinant baculovirus-infected insect cells with androgen, but only when one or both components of the dimer contain the NH_2-terminal domain. Based on the intensities of ARE binding and lack of binding to an ARE half-site, it appears that, unlike the glucocorticoid receptor, AR binds DNA primarily as a dimer. Thus, full-length baculovirus-expressed AR requires intracellular hormone exposure for dimerization and ARE binding to overcome inhibition imposed by the AR NH_2-terminal domain. Antihormones with agonist activity promote dimerization and ARE binding, while a pure antiandrogen blocks AR DNA binding. It is concluded that intramolecular interactions between the NH_2-terminal and steroid-binding domains are regulated by the specificity of hormone binding and modulate receptor dimerization and DNA binding.

The requirement for sufficient amounts of steroid receptors for structure/function studies and *in vitro* transcriptional analysis led to the application of the baculovirus system for overexpression of recombinant receptors. Overexpression of the glucocorticoid (1, 2), mineralocorticoid (3), vitamin D (4), progesterone (5, 6), and androgen (7, 8) receptors was achieved using baculovirus. Expression in baculovirus is advantageous because of the potential for protein processing that occurs in eukaryotic cells. For example, phosphorylation of several steroid receptors was shown to occur in *Spodoptera frugiperda* (Sf9)[1] cells infected with recombinant baculoviral vectors (2, 3, 5), and the expressed receptors were functional in transcriptional activation studies *in vitro* (6). Since transient transfection in monkey kidney COS cells yields androgen receptor (AR) in a predominantly insoluble form in limited amounts, baculovirus was used for overexpression of human AR.

An important question that remains in the functional analysis of AR is whether dimer formation requires hormone binding and occurs in association with DNA binding. Many transcription factors, including members of the steroid receptor family, undergo dimer formation in acquiring high affinity DNA binding (9). Dimerization of steroid receptors was demonstrated in the mobility shift assay using wild type and truncated forms of the progesterone, glucocorticoid (10), and estrogen (11) receptors. Heterodimers were reported for the thyroid hormone, retinoic acid (12–14), and vitamin D receptors. The vitamin D receptor forms heterodimers with a ubiquitous 55-kDa protein that enhances DNA binding (15, 16). Heterodimer formation with coregulatory proteins stabilizes specific DNA interactions of this group of transcriptional regulatory proteins (14).

Studies outlined in this report demonstrate that AR expressed in baculovirus displays high affinity androgen binding, androgen-dependent nuclear translocation, and phosphorylation. Mobility shift assays indicate that full-length baculovirus expressed AR requires intracellular androgen exposure to effect dimer formation and specific DNA binding. A surprising finding was that AR dimerization required androgen exposure in cells only when the NH_2-terminal domain was present. The results indicate that the NH_2-terminal domain acts to inhibit AR dimerization and DNA binding in the unliganded receptor. Furthermore, a pure antiandrogen, hydroxyflutamide, lacks agonist activity because it inhibits AR

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[1] The abbreviations used are: Sf9, *Spodoptera frugiperda*; AR, androgen receptor; Ac*M*NPV, *Autographa californica* nuclear polyhedrosis virus; R1881, methyltrienolone; PCR, polymerase chain reaction; ARE, androgen response element; flutamide, α-α-α-trifluoro-2-methyl-4′-nitro-*m*-propionotoluidide, or 4′-nitro-3′-trifluoromethylisobutyranilide or SCH 13521; hydroxyflutamide, α-α-α-trifluoro-2-methyl-4′-nitro-*m*-lactotoluidide or SCH 16423; cyproterone acetate, 6α-chloro-17α-hydroxy-1α,2α-methylene-4,6-pregnadiene-3,20-dione-17-acetate; RU486, Roussel Uclaf list no. 36,486, mifepristone, 11B-(4-dimethylaminophenyl)-17B-hydroxy-17α-(prop-1-ynyl)-estra-4,9-diene-3-one; DHT, 5α-dihydrotestosterone, 17B-hydroxy-5α-androstan-3-one; AR1–660 or N, expressed fragment of human AR containing amino acids 1–660; AR507–919 or C, expressed fragment of human AR containing amino acids 507–919; m.o.i., multiplicity of infection; kb, kilobase(s); cpm, counts/min; PBS, phosphate-buffered saline; bp, base pair(s).

DNA binding, and thus, represents a new class of antihormones.

EXPERIMENTAL PROCEDURES

Materials—*Spodoptera frugiperda* Sf9 cells derived from the Fall Army worm ovary, *Trichoplusia ni* HighFive® insect cells from cabbage looper eggs, circular baculovirus *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV, 130 kb), and transfection buffer were from Invitrogen Corporation, San Diego CA; pAcC4 baculovirus transfer vector was from Cetus Corp.; Grace medium supplemented with yeastolate (Difco) and lactalbumin hydrolysate (Difco), antibiotics and gentamicin (Life Technology, Inc.) were from the Lineberger Cancer Center, University of North Carolina at Chapel Hill; fetal calf serum was from HyClone Laboratories, Inc.; Trans^{35}-label (L-[^{35}S] methionine, L-[^{35}S]cysteine), 1000 Ci/mmol was from ICN Biomedicals, Inc; phosphate-free and methionine-free, glutamine-supplemented Excell 401 or 400 media were from JRH Biosciences; phosphorus 32-orthophosphoric acid was from Du Pont; [^{3}H]methyltrienolone ([17α-*methyl*-^{3}H]R1881, 80 Ci/mmol) was from Du Pont-New England Nuclear; W-O MAT-AR diagnostic x-ray film was from Kodak; Taq DNA polymerase was from Promega; Sequenase was from U. S. Biochemicals; 0.025-μm cellulose membranes for dialysis was from Millipore; buffers, chemicals, and protease inhibitors were from were Sigma, Fisher, and EM Science.

Construction of Transfer Vectors—Full-length and deletion mutants of human AR were constructed in the 9.1-kb pAcC4 transfer vector. Polymerase chain reaction (PCR) amplification of the human *Nco*I/*Afl*II NH_2-terminal fragment was constructed using Taq polymerase such that the 5′ *Nco*I site contained the starting Met of AR coincident in position with the starting Met of the polyhedron protein. All PCR-amplified regions were verified by sequence analysis using double-stranded sequencing with Sequenase. Triple ligations were performed using the transfer vector restricted with *Nco*I and *Bam*HI, the NH_2-terminal PCR-amplified *Nco*I/*Afl*II fragments, and the *Afl*II/*Bam*HI fragments containing the AR coding sequence. For dimerization studies, two major deletion mutants of human AR were constructed in the pAcC4 transfer vector. Deletion of the steroid-binding domain was achieved by restricting the full-length human AR transfer vector with *Tth*111I and *Xba*I to release the steroid-binding domain; the vector was made blunt ended with T4 DNA polymerase and self-ligated. Deletion of the NH_2-terminal domain was performed by restricting the full-length human AR transfer vector with *Nco*I and *Kpn*I and blunt end-ligated; initiation of translation was from an internal methionine at position 507 (17).

Sf9 Cell Culture and Transfection—Sf9 cells were maintained in 60–100-ml spinner cultures in Grace medium containing 0.33% yeastolate, 0.33% lactalbumin hydrolysate, 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 70 μg/ml gentamicin, and were passaged every 3 days at an initial density of 0.5×10^6 cells/ml. Sf9 cells (4×10^6 cells/6-cm dish) were cotransfected with 1 μg of circular Ac*M*NPV wild type viral DNA and 5 μg of AR transfer vector using 0.75 ml of Invitrogen transfection buffer in the presence of Grace medium that contained 10% serum but lacked supplements. After a 4-h incubation at 27 °C, medium was removed and the cells were incubated in complete Grace medium for 5 days. The transfection supernatants were subjected to serial dilution in 96-well microtiter plates containing 2×10^4 Sf9 cells/well, and recombinant viruses were identified by dot-blot hybridization as previously described (18). The radiolabeled probe was an [α^{32}P]dCTP random prime labeled *Hin*dIII/*Eco*RI DNA fragment of human AR that spans the DNA-binding domain and part of the steroid-binding domain. Three rounds of serial dilution of positive samples were followed by three rounds of plaque purification, where recombinant plaques were visualized using the methyl red overlay technique (19).

Phosphorylation of Baculovirus-expressed AR—Sf9 cells were infected with recombinant human AR baculovirus at a multiplicity of infection (m.o.i.) of 4 using 10^7 cells/10-cm dish for 40–48 h. Medium was replaced with Excell 401 containing L-glutamine but lacking phosphate or L-methionine. After a 20-min incubation at 27 °C, 200 μCi of [^{32}P]orthophosphate or 200 μCi of Trans^{35}S-label was added and incubated for 4 h at 27 °C. Cells were harvested in 4 °C PBS, pH 6.4, microfuged for 30 s, and resuspended in 800 μl of a buffer containing 1 mM EGTA, 1 mM EDTA, 12 mM monothioglycerol, 0.5 M NaCl, 50 mM NaF, 1 mM benzamidine, 1 mM iodoacetamide, 20 mM sodium molybdate, 100 nM R1881, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin, and 50 mM potassium phosphate buffer, pH 7.0. Cells were lysed using three cycles of freeze/thaw and AR immunoprecipitated using antipeptide antibody AR52 and analyzed on an 8% SDS-polyacrylamide gel as previously described (20).

Immunoblot Analysis—In a control experiment, AR expressed from Sf9 cells was analyzed on immunoblots. Sf9 cells were plated at 10^7 cells/10-cm dish, infected with recombinant virus for 40–48 h at m.o.i. 4, and treated for varying times with increasing concentrations of dihydrotestosterone. Cells were washed and harvested in PBS, pH 6.4, at 4 °C, pelleted, and resuspended in high salt extraction buffer (0.5 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 10 mM Tris, pH 7.4, containing the protease inhibitors, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 μM aprotinin, 1 μM leupeptin, 1 μM pepstatin A, and 1 mM benzamidine). Cells were exposed to three cycles of freeze/thaw, incubated on ice for 1 h, microfuged for 30 min, and the supernatant dialyzed against the above resuspension buffer containing 50 mM KCl. The dialyzed supernatant and the pellet were solubilized in SDS sample buffer (2% SDS, 10% glycerol, 10 mM Tris, pH 6.8) and analyzed on an 8% acrylamide minigel. Immunoblots were performed using antipeptide antibody AR52 as previously described (21).

Immunocytochemistry—Sf9 or HighFive® insect cells were infected with recombinant AR baculovirus at m.o.i. 4 in serum free Excell 400 media for 40 h at 27 °C in the presence or absence of 100 nM R1881. Cells were washed in PBS, pH 6.4, and harvested in the same buffer. Cells were smeared on glass slides, air dried, and fixed in 95% alcohol at −20 °C for 10 min. After washing with PBS, pH 7.4, cells were incubated with primary antibody AR32 overnight as previously described (22–24). After incubation, cells were treated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:400; Organon Technika, Cochranville, PA) for 1 h at room temperature. Slides were washed with PBS, pH 7.4, and mounted using 90% glycerol, 1 mM Tris, pH 7.6, and a cover glass placed on the cells. Slides were viewed using a Nikon-UFX-DA fluorescent microscope with a B-2A filter.

Steroid Binding Activity—Steroid binding was performed on intact Sf9 cells infected with human AR recombinant baculovirus using concentrations of [^{3}H]R1881 ranging from 0.1 to 20 nM in the presence and absence of a 100-fold excess of unlabeled hormone. Cells were infected for 48 h at m.o.i. 2, incubated in Excell 400 media for 2 h at 27 °C, and then as a whole cell suspension in Excell 400 medium overnight at 4 °C. Specific binding of [^{3}H]R1881 was undetectable in uninfected cells or larvae. Nonspecific binding of [^{3}H]R1881 ranged from 1–11% of specific binding for 5 and 0.2 nM, respectively.

Gel Mobility Shift DNA Binding Assay—DNA binding was assessed *in vitro* using recombinant full-length or deletion mutant human AR baculovirus expressed in Sf9 cells at m.o.i. 4 in the presence and absence of androgen or various hormones and antihormones. After 48 h of viral infection, cells were incubated an additional 3 h or throughout the entire 48-h infection period at 27 °C with various hormones. Cells were harvested in ice-cold PBS, pH 6.4, and resuspended in TEDG buffer (0.5 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 10 mM Tris, pH 7.4, and protease inhibitors indicated above). Cells were lysed by three cycles of freeze/thaw and incubated on ice for 1 h with vortexing every 15 min. Cell lysates were microfuged for 30 min at 4 °C, and the supernatants were dialyzed for 3 h at 4 °C on a floating 0.025-μm pore, MF-Millipore cellulose membrane filter against TEDG buffer containing 50 mM KCl to reduce the salt concentration, and microfuged for 1 min.

A 27-bp oligonucleotide (AAGCTTAGTACGTGATGTTCTAAGCTT) designated oligonucleotide C has sequence derived from the 0.5-kb first intron region of the rat C3 prostatein gene (25). The underlined regions highlight the androgen response element sequence. In a control study for monomer binding, a 21-bp oligonucleotide (TCGACTGACTCAATTGTTCTG) with flanking *Sal*I sites was used that contained only the right half-site (underlined) of the ARE sequence, shown previously to be the high affinity site for the glucocorticoid receptor (26). Double-stranded oligonucleotides were labeled using [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase. The reaction buffer containing 4 μg of poly(dI-dC), 80 μg of bovine serum albumin, 15–30 μg of infected Sf9 whole cell-extracted protein, 40 mM KCl, 20% glycerol, 0.2 mM EDTA, 2 mM dithiothreitol, and 10 mM Tris, pH 7.5, in a total volume of 20 μl, was preincubated for 15 min on ice. Labeled oligonucleotides (0.2–0.3 ng, 10,000 or 20,000 cpm) were added and incubated for 1 h on ice. The samples were incubated an additional 1 h in the absence or presence of 1 μg of antipeptide antibody AR52 as previously described (24). For mobility shift assays to demonstrate AR dimerization, the amounts of cell extracts ranged from 20–30 μg where half was either the full-length or deletion mutant AR extract. Samples were subjected to electro-

phoresis on nondenaturing 4 or 5% acrylamide gels containing 0.5 × TBE (0.1 mM EDTA, 45 mM boric acid, and 45 mM Tris, pH 8.4). Gels were preelectrophoresed at 100 V for 1 h and samples separated at 150 V at 4 °C for 4–5 h or for 260 V for 2 h (25, 27, 28). Gels were dried under vacuum and autoradiographed by exposure to Kodak X-OMAT film at −70C for 24–48 h.

RESULTS

Construction of Full-length and Truncated Human AR Recombinant Baculoviruses—The full-length coding sequence of human AR was cloned into the *Nco*I and *Bam*HI sites of the polylinker region of pAcC4 baculovirus transfer vector (Cetus). Cloning was achieved by ligation of the restricted vector with a 517-bp PCR amplified NH_2-terminal *Nco*I/*Afl*II AR fragment and a 2.24-kb *Afl*II/*Bam*HI AR fragment from the mammalian expression vectors pCMVhAR (29). As shown in Fig. 1*A*, the vector contains sequences required for homologous recombination with the wild type nuclear polyhedrosis virus from *A. californica*, Ac*M*NPV, and a multiple cloning site that allows placement of the starting AR methionine coincident with that of the polyhedron protein. Two major deletion mutants of human AR were also constructed (see "Experimental Procedures") such that the coding sequence contained the NH_2-terminal and DNA-binding domains (amino acids 1–660, designated AR1–660) or the DNA-binding, hinge, and steroid-binding domains (amino acids 507–919, designated AR507–919) of the 919-amino-acid human AR. Sf9 insect cells were cotransfected with the transfer vectors and wild type Ac*M*NPV DNA. Viral stocks were collected from infected cells and recombinant plaques enriched by serial dilution and dot-blot hybridization. Recombinant plaque purification was achieved by visual screening for absence of polyhedrin-containing occlusion particles. Several plaques for each construction were selected for further characterization.

Properties of Baculovirus-expressed AR from Sf9 Cells—Cultured Sf9 cells maximally express AR within 30–48-h post infection at m.o.i. 2–10 and produce a 118 ± 4-kDa protein that comigrates on an immunoblot with AR expressed in COS cells (see Fig. 6). AR displayed high affinity, saturable binding of [^{3}H]R1881 in a whole cell binding assay, with approximately 6 pmol/mg protein, and the apparent binding affinity constant, K_d 0.3 ± 0.2 nM (Fig. 1, *B* and *C*). A Hill coefficient of 1.2 suggested minimal cooperative androgen binding.

AR undergoes androgen-dependent translocation from the cytoplasm to the nucleus in insect cells (Fig. 2). No fluorescent immunostaining was detected in uninfected Sf9 (Fig. 2*A*) or *T. ni* cells (Fig. 2*D*) in agreement with the lack of endogenous AR. In the presence of 50 nM R1881, AR staining was predominantly nuclear (Fig. 2, *B* and *E*), particularly within the virogenic stroma, a region of condensed chromatin within insect cell nuclei. AR distributed in the cytoplasm of insect cells in the absence of androgen (Fig. 2, *C* and *F*). Thus, baculovirus-expressed AR undergoes androgen-dependent targeting to the nucleus in insect cells.

AR phosphorylation was evident when baculovirus-infected Sf9 cells were incubated with [^{32}P]orthophosphate. Androgen caused an increase in both receptor phosphorylation and stabilization during a 3-h incubation at 27 °C similar to that previously reported for receptor expressed in COS cells (20), indicating that AR undergoes post-translation modification in insect cells and is a phosphoprotein (data not shown).

Intracellular Hormone Dependence of in Vitro AR DNA Binding—The intracellular androgen dependence of specific AR DNA binding was determined in an *in vitro* DNA binding assay using a ^{32}P-labeled 27-bp oligonucleotide containing a full ARE sequence (see "Experimental Procedures"). A faint

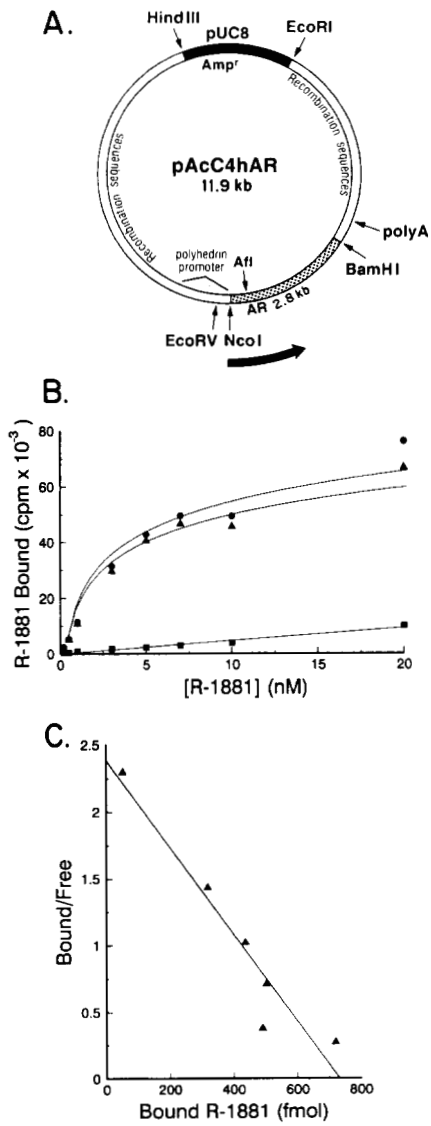

FIG. 1. **Recombinant baculovirus transfer vector and binding studies.** *A*, Schematic diagram of the AR baculovirus transfer vector. The pAcC4 transfer vector from Cetus Corporation contained sequences for homologous recombination with the nuclear polyhedrosis wild type Ac*M*NPV baculovirus and a multiple cloning site following the polyhedrin promoter. An *Nco*I site was coincident with the starting Met of the polyhedrin protein. Full-length human AR 2.8-kb coding sequence was cloned into the *Nco*I/*Bam*HI site in the polylinker region. The 5′ end of the human AR was constructed by PCR amplification using a 5′ primer containing the *Nco*I site and the 5′ coding sequence of AR and the 3′ primer position 3′ of the internal *Afl*II site. A triple ligation led to the final construct of 11.9 kb. *B* and *C*, saturation binding and Scatchard plot analysis of baculovirus expressed human AR binding of [^{3}H]R1881 in Sf9 cells. Sf9 cells were infected with recombinant human AR baculovirus at m.o.i. 2 as described under "Experimental Procedures." Cells were harvested, washed in PBS, pH 6.4, and aliquoted at approximately 2 × 10^5 cells/assay. Intact cells were incubated at increasing concentrations of [^{3}H]R1881 in the presence or absence of unlabeled R1881 overnight at 4 °C, washed three times in PBS, pH 6.4, the pellet solubilized in 2% SDS, 10% glycerol, and 10 mM Tris, pH 6.8, and radioactivity determined by scintillation counting. Specific binding represented the difference between counts/min bound in the presence and absence of unlabeled hormone. Illustrated is total (●), nonspecific (■), and specific binding (▲) (*B*) and a Scatchard plot of specific binding (*C*). Apparent binding constant K_d 0.3 ± 0.2 nM K_d; Hill coefficient 1.2.

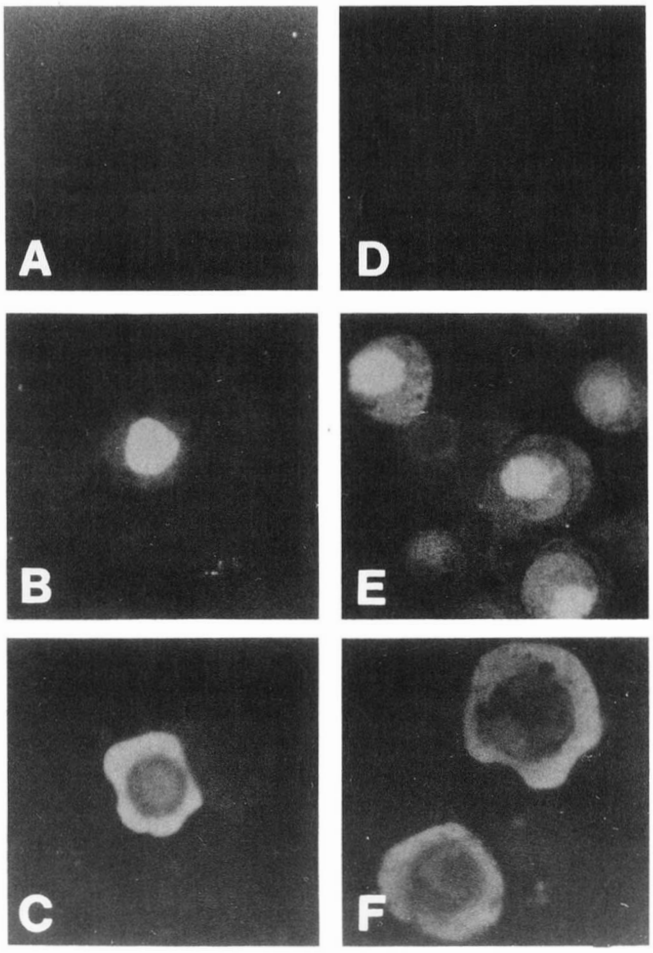

FIG. 2. **Immunofluorescent localization of human AR in Sf9 and high five cells infected with recombinant human AR baculovirus.** Sf9 cells and *T. ni* HighFive® cells were infected with recombinant human AR baculovirus as described under "Experimental Procedures." Immunofluorescent staining was performed using rabbit antipeptide IgG AR32 specific for AR. Immunostaining is shown for uninfected Sf9 cells (*A*), Sf9 cells infected with recombinant human AR baculovirus incubated in the presence (*B*) or absence (*C*) of 100 nM R1881; *T. ni* high five cells uninfected (*D*), or infected with recombinant human AR baculovirus incubated in the presence (*E*) or absence (*F*) of 100 nM R1881. Magnification × 500.

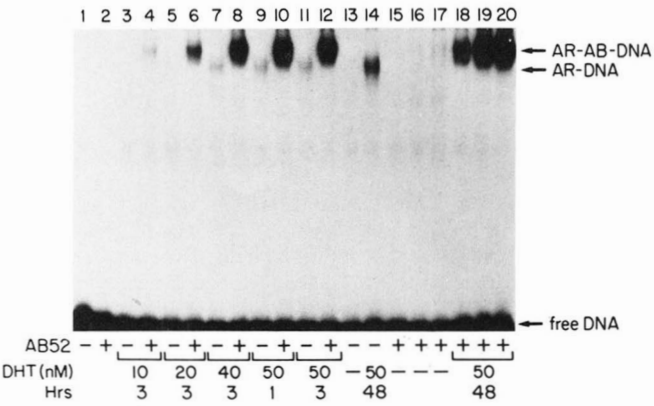

FIG. 3. **Androgen dependence of baculovirus expressed human AR binding to androgen response element DNA.** Sf9 cells were uninfected with virus (*lanes 1* and *2*) or infected with the full-length human AR recombinant baculovirus (*lanes 3–20*) and incubated for varying times (*Hrs*) with increasing amounts of DHT from 10–50 nM as indicated. Cells were extracted in high salt containing buffer as described under "Experimental Procedures" and 10 μg (*lanes 15* and *18*), 15 μg (*lanes 1–12*), 20 μg (*lanes 13, 14, 16,* and *19*), or 30 μg (*lanes 17* and *20*) of whole cell extract was added to each 20 μl of binding reaction. Some reactions contained in addition 1 μg of AR52 antipeptide IgG as indicated (+ *AB52*). The ^{32}P-labeled androgen response element DNA (10,000 cpm/reaction) was added as described under "Experimental Procedures." Two prominent bands were detected including the AR-androgen response element DNA complex (*AR-DNA*) and that complexed with the AR antibody (*AR-AB-DNA*). The *band* at the *bottom* of the gel represents unbound ^{32}P-labeled ARE DNA.

nonspecific band was observed when extracts from uninfected Sf9 cells were combined with ARE DNA, a protein-DNA complex that was unaffected by AR antibody addition (Fig. 3, *lanes 1* and *2*). No additional DNA complexes were observed using extracts from cells infected with recombinant AR baculovirus in the absence of androgen (Fig. 3, *lane 13*). However, addition of dihydrotestosterone to cells expressing AR resulted in a dose-dependent formation of an AR·DNA complex that increased in intensity as the concentration increased from 10 to 50 nM dihydrotestosterone (Fig. 3, *lanes 3, 5, 7, 9,* and *11*). The most intense band observed in the absence of antibody occurred following 24–48 h of treatment with 50 nM dihydrotestosterone (Fig. 3, *lane 14*). Unlabeled ARE DNA in 100-fold excess competed for AR·DNA complex formation (data not shown) indicating that the DNA binding was specific.

Dose-dependent androgen-induced increases in AR·DNA complex formation were also evident in the presence of the AR antipeptide antibody AR52. In the absence of cell exposure to dihydrotestosterone, only weak intensity bands were observed with full-length AR when tested with AR52 IgG at increasing concentrations of Sf9 cell extracts (Fig. 3, *lanes 15–17*). Addition of 50 nM dihydrotestosterone to the Sf9 cell incubations 48 h prior to harvest and AR52 IgG to the binding reactions caused up to a 120-fold increase in band intensity (Fig. 3, *lanes 18–20*). A dose-dependent increase in complex formation with increasing androgen concentrations was also observed in the presence of AR antibody (Fig. 3, *lanes 4, 6, 8, 10,* and *12*). Addition of AR52 IgG potentiated androgen-dependent AR-DNA binding by at least 11-fold after cell incubation times of 1–3 h with androgen treatment. Enhancement of AR DNA binding by antibody may reflect antibody stabilization of AR dimerization (see below). Immunoblot analysis revealed similar amounts of AR protein in extracts from cells incubated in the absence and presence of increasing androgen concentrations for 3 h. After 30 h in the presence of androgen, some increase in AR protein was noted (data not shown).

Steroid Specificity of AR DNA Binding—We established whether exposure of Sf9 cells expressing AR to nonandrogenic hormones could effect AR DNA binding. As shown in Fig. 4, hormone-induced ARE binding reflected the ability of that hormone to activate a reporter gene previously reported (20). Strongest DNA binding was noted with the androgens, R1881, dihydrotestosterone, and testosterone (Fig. 4, *lanes 1, 3,* and *5*), and the addition of AR antibody further enhanced DNA binding (Fig. 4, *lanes 2, 4,* and *6*). Estrogen and progesterone promoted weak DNA binding only apparent in the presence of AR antibody (Fig. 4, *lanes 7–10*). Weakest activities were noted with dexamethasone and flutamide which do not bind AR with significant affinity at the concentrations tested. Somewhat to our surprise, the antiandrogen cyproterone acetate (Fig. 4, *lanes 13* and *14*) and the antiprogestin RU486 (Fig. 4, *lanes 19* and *20*) promoted relatively strong AR DNA binding, particularly in the presence of antibody. Both of these antihormones were shown previously to display agonist activity (20).

Of particular interest was hydroxyflutamide, since it binds

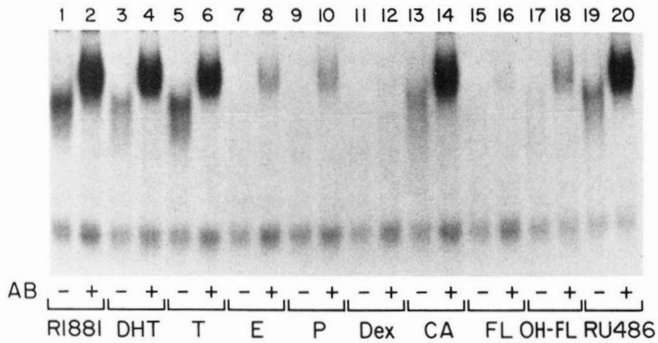

FIG. 4. **Steroid specificity of AR DNA binding.** Sf9 cells expressing AR were incubated with 50 nM R1881 (*lanes 1* and *2*), DHT (*lanes 3* and *4*), or testosterone (*T*, *lanes 5* and *6*), or 100 nM estradiol (*E*, *lanes 7* and *8*), progesterone (*P*, *lanes 9* and *10*), dexamethasone (*Dex*, *lanes 11* and *12*), cyproterone acetate (*CA*, *lanes 13* and *14*), flutamide (*FL*, *lanes 15* and *16*), hydroxyflutamide (*OH-FL*, *lanes 17* and *18*), or RU486 (*lanes 19* and *20*). *Even numbered lanes* had 1 μg of AR52 antibody added during the DNA binding reactions.

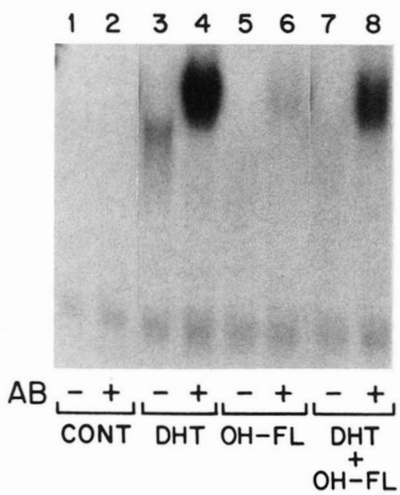

FIG. 5. **Inhibition of androgen-induced AR-DNA binding by hydroxyflutamide.** Sf9 cells were left uninfected (*lanes 1* and *2*) or were infected with recombinant human AR baculovirus (*lanes 3–8*). Sf9 cells expressing AR were incubated for 42 h with 50 nM DHT (*lanes 3* and *4*), 100 nM hydroxyflutamide (*OH-FL*, *lanes 5* and *6*), or simultaneously with 50 nM DHT and 500 nM OH-FL (*lanes 7* and *8*). *Even numbered lanes* contain cell extracts in which AR52 antibody (1 μg) was added during the DNA binding reaction (*AB +*). *CONT*, control uninfected cell extracts.

AR with moderate affinity, causes nuclear transport, but fails to transactivate an androgen-responsive reporter gene (20). Hydroxyflutamide was a poor activator of AR DNA binding (Fig. 4, *lanes 17* and *18*). Furthermore, including hydroxyflutamide together with DHT in the cell incubations markedly reduced AR DNA binding (Fig. 5). These results suggest that the antagonist activity of hydroxyflutamide results from its inability to invoke the necessary intracellular changes in AR to permit DNA binding.

Receptor Dimerization—AR dimer formation was investigated using two deletion mutants of human AR expressed in baculovirus: AR1–660 contained the NH_2-terminal and DNA-binding domains and AR507–919 contained the DNA-, hinge, and steroid-binding domains (diagrammed in Fig. 7). Expression of the deletion mutants from baculovirus was verified by immunoblot analysis, revealing truncated receptor forms of 85 ± 3 kDa (AR1–660, Fig. 6, *lane 5*, *N*) and 43 ± 1.5 kDa (AR507–919, Fig. 6, *lane 6*, *C*) relative to 118 ± 4 kDa full-length AR (Fig. 6, *lane 4*). The smaller sized bands reflect limited proteolytic cleavage prior to analysis. Dimerization was examined using ^{32}P-labeled ARE DNA described above, and initially, receptors extracted from cells exposed to dihydrotestosterone.

Incubation of AR with ARE DNA resulted in a slowly migrating AR·DNA complex (Fig. 7, *lane 7*). A faster migrating complex was observed with AR507–919 (Fig. 7, *lane 3*), and AR1–660 formed a weak DNA complex migrating similar to full-length AR-DNA (Fig. 7, *lane 5*). Addition of antipeptide antibody AR52 caused a further shift in each band, verifying the presence of AR in the protein-DNA complexes (Fig. 7, *lanes 4*, *6*, and *8*). No specific bands were observed using extracts of uninfected cells in the presence or absence of antibody (Fig. 7, *lanes 1* and *2*).

AR dimerization was evident in the mobility shift assay using combinations of truncated and full-length receptors. A major band of intermediate migration formed between deletion mutants AR1–660 and AR507–919 (Fig. 7, *lanes 10–12*). The intermediate band was 39- and 6-fold greater intensity than either mutant alone, respectively, suggesting a synergistic increase in DNA binding with dimerization. About 15% residual fragment AR507–919-DNA complex migrated in its original position (Fig. 7, *lane 12* compared with *lane 11*). Dimer formation between the dissimilar deletion mutants was therefore stronger than between either mutant alone.

Combining full-length AR and AR507–919 also resulted in strong dimer formation, with an intermediate band (Fig. 7, *lanes 14–16*) migrating slightly slower than the AR1–660-AR507–919 DNA complex (*lane 12*). Residual AR·DNA complexes remained detectable at their original positions of migration. The ratio of intensity of the three bands was 2:7:1 (Fig. 7, *lane 16*), corresponding to full-length AR, dimer, and AR507–919. Thus, dimerization between full-length AR and AR507–919 was preferred to dimerization of either with itself, assuming that AR·DNA complexes observed with each fragment alone represents dimerization (see below).

Dimerization of full-length AR and AR1–660 results in inhibition of DNA binding of full-length AR (Fig. 7, *lanes 18–20*). The same low level of DNA binding was observed with full-length AR and AR1–660 as with AR1–660 alone, suggesting that, in the absence of one or both hormone-bound ligand-

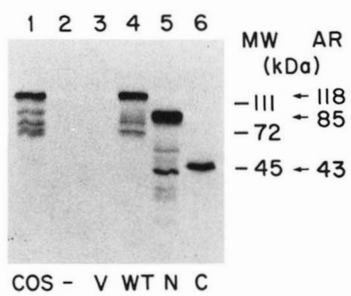

FIG. 6. **Immunoblot analysis of full-length and deletion mutants expressed from baculovirus in Sf9 cells.** Cells plated at 10^7/10-cm dish were uninfected (*lane 2*), or infected for 48 h with wild type virus lacking AR sequence (*lane 3*), full-length human AR recombinant baculovirus (*lane 4*), carboxyl-terminal deletion mutant AR1–660 (amino acids 1–660 *lane 5*, designated *N*), NH_2-terminal deletion mutant AR507–919 (amino acids 507–919, *lane 6*, designated *C*). Cells were harvested in PBS, pH 6.4, extracted in high salt buffer (0.5 M NaCl, 1 mM EDTA, 10% glycerol, 10 mM Tris, pH 7.4, containing 0.5 mM phenylmethylsulfonyl fluoride, 0.1 μM aprotinin, 1 μM leupeptin, 1 μM pepstatin A, 1 mM benzamidine) and subjected to three cycles of freeze/thaw. Aliquots were analyzed on immunoblots as described under "Experimental Procedures." Full-length AR from transiently transfected COS cells (*COS*, *lane 1*) prepared as previously described (17) served as a positive control for full-length human AR.

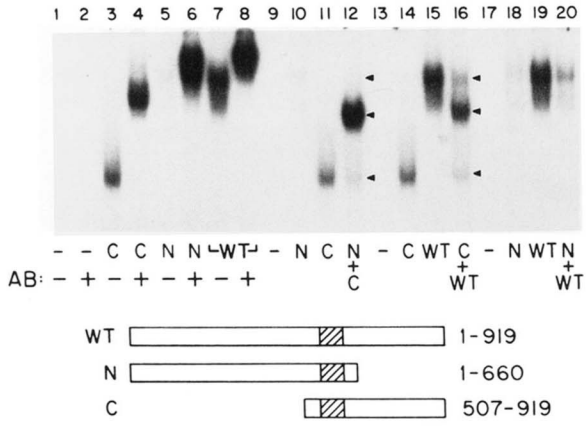

FIG. 7. **Dimerization of human AR.** Receptor dimerization was determined in the DNA mobility shift assay using Sf9 cells infected with recombinant baculovirus encoding either full-length AR (*WT*), AR1–660 (amino acids 1–660, *N*) or AR507–919 (amino acids 507–919, *C*) as indicated. Dihydrotestosterone (50 nM) was added to the media at the time of transfection, 48 h prior to cell harvest and extraction. Whole cell extracts (20 μg total protein/reaction) were prepared as described under "Experimental Procedures" and incubated in the DNA binding assay using ^{32}P-labeled androgen response element DNA; reactions with different AR fragments contained 10 μg of protein from each cell extract. Sf9 cell extracts were from uninfected cells (*lanes 1, 2, 9, 13,* and *17*) or from cells infected with the full-length AR (*lanes 7, 8, 15, 16, 19,* and *20*), AR1–660 (*lanes 5, 6, 10, 12, 18,* and *20*), and AR507–919 (*lanes 3, 4, 11, 12, 14,* and *16*). The presence of AR in the DNA complex was established by adding to some reactions 1 μg of AR52 IgG antibody (*lanes 2, 4, 6,* and *8*). *Arrows* highlight the major DNA-protein complexes in *lanes 12* and *16*. Below is a schematic diagram illustrating regions of AR expressed by the wild type and mutant vectors.

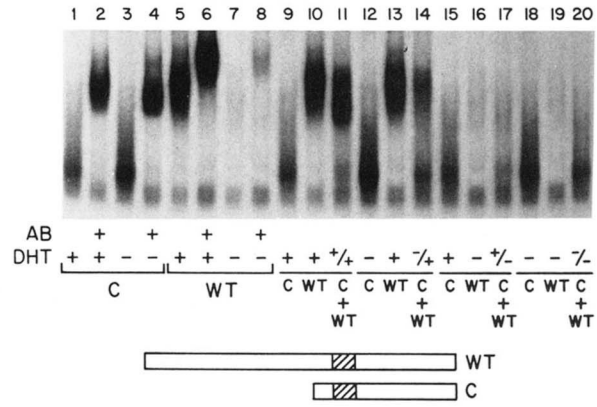

FIG. 8. **Requirement for intracellular androgen exposure in AR dimerization.** Dimerization was studied in the DNA mobility shift assay using AR507–919 (*C*) and full-length AR (*WT*). Cells expressing AR507–919 or full-length AR were incubated in the presence or absence of 50 nM dihydrotestosterone (*DHT*) as indicated: +/+, C and WT treated with hormone, −/+, only WT treated with DHT; +/−, only C treated with DHT; −/−, neither treated with hormone. Cell extracts were combined in the DNA binding reaction as indicated in *lanes 11, 14, 17,* and *20* and assayed as described under "Experimental Procedures" using the C3 ARE [^{32}P]oligonucleotide. *Lanes 2, 4, 6,* and *8* contained samples to which AR antibody AR52 was added (*AB +*). Below is a schematic diagram of AR507–919 and full-length AR coding regions.

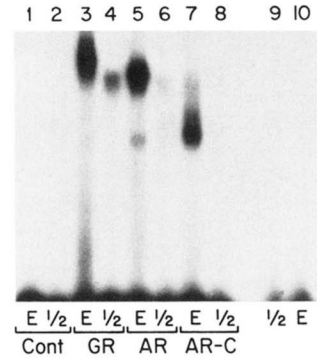

FIG. 9. **Absence of AR monomer binding to a right half-site ARE.** Sf9 cells expressing full-length glucocorticoid receptor (*GR, lanes 3* and *4*), full-length AR (*lanes 5* and *6*), and AR507–919 (*AR-C, lanes 7* and *8*) were treated with either dexamethasone for glucocorticoid receptor as previously described (1) or with 50 nM R1881 for 40 h. High salt cell extracts prepared as described under "Experimental Procedures" were incubated in the presence of either 20,000 cpm/reaction of the full ARE [^{32}P]oligonucleotide (designated *E, lanes 3, 5,* and *7*) or with 50,000 cpm/reaction [^{32}P]oligonucleotide containing only the right half-site ARE (TGTTCT) (designated *1/2, lanes 4, 6,* and *8*). Full and half-site labeled oligonucleotides were assayed in addition with Sf9 cell extracts without prior viral infection (*lanes 1* and *2*) or with no protein addition to the DNA binding reaction (*lanes 9* and *10*). DNA binding assays were performed as described under "Experimental Procedures," and the reaction mixtures were separated on a 4% nondenaturing agarose gel at 4 °C at 170 V for 2 h. The *band* at the *bottom* of the gel represents unbound ^{32}P-labeled DNA. *CONT*, control uninfected cell extracts.

binding domains, the two NH_2-terminal domains in the AR dimer inhibit DNA binding.

Intracellular Androgen Dependence of in Vitro Dimerization—We next determined whether androgen exposure of Sf9 cells expressing AR was required for AR507–919 dimerization with itself or with full-length AR. AR507–919 extracted from cells untreated with androgen produced a strong migrating DNA-protein complex (Fig. 8, *lane 3*) which migrated slightly faster than when extracted from androgen treated cells (Fig. 8, *lane 1*). The strong band intensities suggest that AR507–919 is capable of binding DNA independent of androgen exposure in Sf9 cells. The slight difference in migration may result from conformational differences in AR507–919 in the presence or absence of androgen binding. Both AR507–919-DNA complexes shifted with antibody (Fig. 8, *lanes 2* and *4*).

Further evidence for dimerization of AR507–919 independent of androgen was obtained using an oligonucleotide containing a single right half-site ARE consensus sequence TGTTCT (see "Experimental Procedures"). A similar oligonucleotide was used previously to demonstrate monomer binding of the glucocorticoid receptor (30). Little or no detectable bands were observed using full-length AR507–919 with the right half-site oligonucleotide (Fig. 9, *lanes 6* and *8*). In a control experiment, full-length glucocorticoid receptor expressed from baculovirus displayed binding to both the half-site and full ARE (Fig. 9, *lanes 3* and *4*). Monomer binding of the glucocorticoid receptor was also evident by the change in mobility of DNA complexes formed between the dissimilar oligonucleotides (Fig. 9, *lanes 3* and *4*). The results suggest that AR, either in its full-length or NH_2 terminally truncated form, binds DNA primarily as a dimer and that ARE binding of AR507–919 reflects dimer binding. Furthermore, AR507–919 dimerization and DNA binding occurs independent of androgen exposure.

Full-length AR dimerization with AR507–919 was examined where either one or both were exposed to androgen in Sf9 cells. As shown above, dimerization occurred when both full-length AR and AR507–919 were exposed to androgen (Fig. 8, *lanes 9–11*). However, if either was untreated with androgen, no dimerization was observed (Fig. 8, *lanes 12–20*). Thus, unlike AR507–919 dimerization with itself, dimerization of full-length AR with AR507–919 required that each be exposed to androgen in Sf9 cells. The results support the hypothesis that the AR NH_2-terminal domain inhibits dimer

formation in the absence of androgen and that androgen treatment relieves this inhibition.

Since AR1–660 and AR507–919 form a strong dimer band (see Fig. 7, *lane 12*), it was important to establish whether AR507–919 in this complex required androgen exposure as it did in dimerization with full-length AR but not with itself. Fig. 10 shows that androgen exposure of AR507–919 in Sf9 cells is required for dimerization with AR1–660. The results reaffirm the inhibitory influence of the NH_2-terminal domain in dimer formation, even in a complex where one fragment contains the steroid-binding domain and the other contains the NH_2-terminal domain. The results raise the possibility that regions required for strong dimer formation are located within the domains of these dissimilar AR fragments.

Finally, we tested the hormone specificity of dimerization. In control experiments in the absence of hormone, full-length AR fails to bind the ARE, AR507–919 binding is strong and dimerization between them is undetectable (Fig. 11, *lanes 2–4*). Strong dimerization occurs with R1881 and DHT (Fig. 11, *lanes 5–10*). Dimers are also detected with cyproterone acetate (*lane 13*) and RU486 (*lane 16*), but DNA binding and dimerization fails to occur with hydroxyflutamide (*lanes 17–19*). It is noteworthy that the migration of AR507–919 changes markedly depending on hormone treatment. For example, AR507–919 migration with dihydrotestosterone (Fig. 11, *lane 9*) differs from that with R1881 (*lane 6*) but is similar to that observed in the absence of hormone (*lane 3*). Also, it is important to note that hydroxyflutamide inhibits ARE binding of both full-length AR (*lane 17*) and AR507–919 (compare *lanes 3* and *18*). The change in AR507–919 DNA complex migration likely reflects changes in receptor conformation resulting from hormone binding.

DISCUSSION

Overexpression of recombinant steroid receptors provides an important approach with which to determine the molecular properties of this family of transcriptional regulatory proteins. By the criteria of androgen binding, subcellular localization,

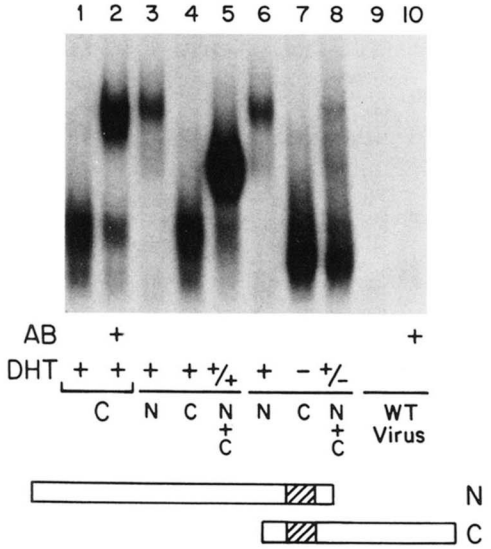

FIG. 10. **Androgen dependence of AR dimerization using the AR1–660 and AR507–919 deletion mutants.** Sf9 cells expressing AR1–660 (*N*) or AR507–919 (*C*) were incubated in the presence (+) or absence (−) of 50 nM DHT as indicated: +/+, both treated with DHT; +/−, only AR1–660 treated with DHT. Cell extracts were combined in the DNA binding reaction as indicated in *lanes 5* and *8*. *Lanes 9* and *10* contain extracts from cells infected with the wild type baculovirus. Cell extracts in *lanes 2* and *10* had AR52 antibody added in the DNA binding reaction (*AB* +).

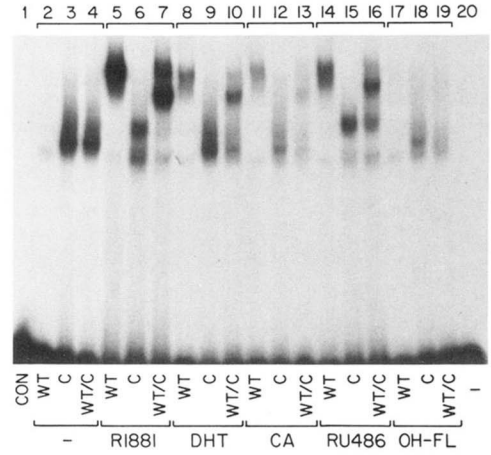

FIG. 11. **AR dimerization in the presence of androgens and antihormones.** Sf9 cells were infected with recombinant baculovirus encoding either full-length AR (*WT*) or AR507–919 (*C*) as indicated. Cells were incubated with 50 nM R1881, 50 nM DHT, 100 nM cyproterone acetate (*CA*), 100 nM RU486, or 100 nM hydroxyflutamide (*OH-FL*) as indicated. Whole cells extracts (20 μg of total protein/reaction) were incubated with ^{32}P-labeled ARE oligonucleotide in the DNA binding assay. Reactions containing two AR fragments contained 10 μg of total protein from each cell extract treated with or without hormone. *Lane 1* contains control cell extract from Sf9 cells without prior viral infection (*CON*). *Lane 20* contains the ^{32}P-labeled oligonucleotide to which no protein was added in the DNA binding reaction. The radiolabeled band at the bottom of the gel represents unbound labeled ARE DNA.

and phosphorylation, a functional AR is expressed in Sf9 cells from baculovirus. It was determined that the AR requires prior intracellular exposure to androgen in order to acquire the ability to bind an ARE sequence *in vitro*. The steroid specificity of this hormone-dependent intracellular change in AR correlated with agonist activity demonstrated previously for a variety of hormones (20). Particularly noteworthy was the differential response of the antihormones: those that display agonist activity in transient cotransfection assays (cyproterone acetate and RU486) induce AR DNA binding, while the pure antiandrogen, hydroxyflutamide, which lacks agonist activity, failed to potentiate AR DNA binding, and, in fact, inhibited AR DNA binding when added together with androgen. The antagonistic activity of hydroxyflutamide results, therefore, from its failure to induce changes in AR necessary for DNA binding.

To investigate further the molecular basis for the steroid-mediated changes in AR required for DNA binding, two AR deletion mutants were expressed from baculovirus in Sf9 cells. The results indicate that AR undergoes dimerization in association with ARE binding, and, as a monomer, fails to bind the ARE. Furthermore, by using different combinations of full-length and AR deletion mutants, it was established that dimerization was dependent on intracellular hormone exposure in order to overcome inhibition created by the AR NH_2-terminal domain. This conclusion was supported by the observation that deletion mutant AR507–919, which lacked the NH_2-terminal region, dimerized and bound DNA efficiently in the absence of hormone, and that the presence of the NH_2-terminal domain blocked this hormone independent DNA binding. Finally, it is speculated that the hormone specificity of DNA binding reflects the ability of a given hormone to promote conformational changes in AR that overcome NH_2-terminal domain inhibition.

The critical role of hormone in steroid receptor action is believed to be activation of receptors to a DNA binding state. The demonstration of hormone-dependent DNA binding is

sometimes complicated by isolation procedures that often cause artificial receptor activation so that receptors no longer require hormone for high affinity DNA binding. Sf9 cells expressing AR must be exposed to androgen intracellularly in order to render the receptor capable of binding DNA *in vitro*. In a recent study using baculovirus-expressed AR, binding to androgen response element DNA occurred independent of androgen exposure (7). An explanation for the differing results is that the AR used in the previous report was extracted using denaturing conditions and was subsequently renatured. Ligand-dependent DNA binding was reported, however, for baculovirus-expressed human progesterone receptor (5, 6).

Antihormones bind AR with moderate affinity, cause nuclear transport (20), but, as shown in this report, differ in their ability to induce DNA binding. These observations suggest that hormone binding may impose different conformations on the receptor, which either allow or disallow DNA binding. That steroid hormones play a major role in receptor conformation is supported by the recent study of Allan *et al.* (31) where proteolytic cleavage products differed in size after hormone and antihormone exposure of the progesterone receptor. It was proposed that distinct receptor conformations are induced by hormone and antihormone binding. The primary site for conformational modification was thought to be the carboxyl-terminal tail (32). A monoclonal antibody raised against the 14 terminal amino acids of the progesterone receptor only recognized the progesterone receptor when unliganded or bound to the steroid antagonist, RU486, suggesting that agonist binding induces a unique receptor conformation (33). Our studies using hydroxyflutamide support the formation of an improper receptor conformation that interferes with DNA binding. This was evident even with the NH_2 terminally truncated mutant AR507–919, that without hormone, displayed strong DNA binding, but with hydroxyflutamide, showed inhibition of DNA binding.

Further evidence for the importance of the carboxyl-terminal tail in conformational effects that differentiate agonist and antagonist activity comes from a switch in the activity of hydroxyflutamide from antagonist to agonist in an AR mutant. Agonist activity of hydroxyflutamide is undetectable with wild type AR but becomes significant with an AR mutant containing a single base mutation at amino acid residue 877, changing threonine to alanine, in the carboxyl-terminal region of the AR steroid-binding domain (34, 35). It could be speculated that the single residue change contributed to a change in AR conformation when bound to hydroxyflutamide which potentiated DNA binding and thus, transactivation. Similarly, a single amino acid change in the progesterone receptor disallowed antagonist RU486, but not agonist binding (36).

Alternative mechanisms for the molecular basis of steroid antagonism include reduced nuclear translocation with concomitant inhibition of 10 S to 4 S conversion, as shown for the glucocorticoid receptor (37). All of the antihormones tested in the present report, including hydroxyflutamide, caused nuclear transport of AR (20) and preliminary studies with baculovirus-extracted receptor indicate that hormone- and nonhormone-treated receptors migrate as 5–6 S complexes on sucrose gradients. Studies on RU486 interaction with the human progesterone receptor indicate that binding to a specific response element was indistinguishable from that with the synthetic agonist, R5020 (38). Although the progesterone receptor binds its response element when associated with RU486, it apparently binds with a somewhat altered conformation reflected by differences in migration on sucrose gradients and in DNA mobility shift assays; failure to transactivate results therefore from structural alterations (39). Using deletion mutants of the rabbit progesterone receptor, it was demonstrated that RU486-bound receptors interact with the same response element (40). In agreement with these studies, the AR-RU486 dimer appeared to migrate with slightly altered mobility. These studies support the important role of receptor conformation mediated by steroid binding in transcriptional activation. They point out that the molecular basis of hydroxyflutamide antagonism differs from that of most antihormones reported thus far, which bind with high affinity to their respective hormone response elements, as recently summarized (41, 42). Hydroxyflutamide therefore belongs to a new class of "pure" antagonists that lack agonist activity due to their inability to promote specific DNA binding.

AR dimerization and the inhibitory role played by the NH_2-terminal domain was revealed using full-length and deletion baculovirus expressed mutants in the DNA mobility shift assay. AR1–660, that contains the NH_2-terminal and DNA-binding domains, and AR507–919, the DNA- and steroid-binding domains, showed strong hormone-dependent dimerization. Thus, a major dimerization domain may reside within their overlapping regions in the DNA binding and hinge regions. Alternatively, dimerization may involve distinct regions in each of the dimer components. The DNA-binding domain of the glucocorticoid receptor contains a dimerization region (10, 11) within a so-called D box of the second zinc finger (43, 44). Crystal structure analysis of the glucocorticoid receptor DNA-binding domain was linked directly to DNA binding-induced dimerization (45). A major dimerization domain of the estrogen receptor occurs within the steroid-binding domain (46), and the ligand-binding domain was proposed as a site for a major dimer interface that interacts with the same region in the dimer component (44). Such a mechanism does not comply with our results with AR. A dimerization region within the steroid-binding domain must necessarily interact with a region outside the steroid-binding domain in the other dimer component.

The intensity of DNA-receptor complex formation between AR1–660 and AR507–919 deletion mutants exceeded that observed with AR1–660 and the full-length receptor, both of which contain the NH_2-terminal region. The results suggest that the AR NH_2-terminal domain within the dimer weakens dimer strength. The results raise the possibility that efficient dimerization and DNA binding might occur when AR heterodimerizes with another protein. In this regard, it was recently reported that a truncated form of AR expressed in *Escherichia coli* complexes with an AR accessory factor that potentiates DNA binding (47).

After short incubation with androgen (1–3 h), AR displayed relatively weak DNA binding that was potentiated by antibody. AR antibody did not enhance DNA binding using extracts of cells untreated with androgen, even though the extraction buffer contained high salt. Furthermore, more prolonged androgen exposure of Sf9 cells (48 h) caused AR to bind DNA strongly independent of antibody. It was noted that addition of androgen to the *in vitro* DNA binding reaction without prior exposure of Sf9 cells was ineffective in promoting DNA binding, even though specific high affinity androgen binding was measured. These results, taken together, indicate that intracellular changes occur in AR in response to androgen that enable the receptor to bind DNA. The molecular basis for this sequential, time-dependent acquisition of DNA binding remains to be determined. Post-translational modification of AR such as phosphorylation occurs in Sf9 insect cells and could theoretically have a role in the induction of strong DNA binding. Antibody may stabilize receptor dimerization or a

conformation conducive to DNA binding.

The results indicate that AR activation in Sf9 cells requires hormone- and time-dependent intracellular processes. Certain pure antiandrogens lack agonist activity because they fail to promote dimerization and DNA binding. Failure to promote DNA binding likely results from an altered conformation imposed on the ligand-binding domain. The AR NH_2-terminal domain blocks DNA binding in the absence of the appropriate ligand by interfering with receptor dimerization.

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